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A COMPETITION BINDING ASSAY FOR DETERMINATION OF THE INOSITOL (1,4,5)-TRISPHOSPHATE CONTENT OF HUMAN LEUCOCYTES

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SUMMARY: We developed a competition binding assay for estimation of the intracellular inositol (1,4,5)-trisphosphate (Ins(1,4,5)P₃) and optimized it for the measurement of the Ins(1,4,5)P₃ content of human blood leucocytes. The present method is considerably cheaper and requires five times fewer cells than the commercial Ins(1,4,5)P₃ kit. The mean Ins(1,4,5)P₃ content of human blood monocytes, granulocytes, and lymphocytes amounted to 3.3±1.2 μM, 3.1±1.4 μM, and 4.6±1.5 μM, respectively. After stimulation with formyl-methionyl-leucyl-phenylalanine (f-MLP) the Ins(1,4,5)P₃ content of human granulocytes and monocytes increased 2-3 times within 10 sec and then gradually decreased, returning to basal values at 60 sec. Lymphocytes did not respond to f-MLP with an increase in their Ins(1,4,5)P₃ content. © 1990 Academic Press, Inc.

Quantification of the cellular Ins(1,4,5)P₃ content is usually performed in cells loaded with ³²[P]-labeled phosphate or myo-(2-³[H])-inositol (2-³[H]-Ins) until an equilibrium between radiolabeled phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P₂) and radiolabeled Ins has been reached (1-3). It is assumed that all pools of inositolphosphates have then been labeled to equilibrium. After loading, the inositolphosphates are extracted from the cells and analysed by chromatography (4-7). These procedures have certain drawbacks: 2-³[H]-Ins is poorly taken up by cells and chromatographic analysis is complex and time-consuming. Several methods have been developed to assess the Ins(1,4,5)P₃ content of cells without the introduction of labeled phosphate or Ins (8-11). Only the competition binding assay (9) does not rely on chromatographic analysis of the extracted inositolphosphates. This assay takes advantage

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Abbreviations used: InsP₁₋₆, myo-inositol mono-, bis-, tris-, tetrakis-, pentakis-, hexakis-phosphate, with positional isomerism of phosphoesters as indicated in parentheses; GroInsP₁, glycerophosphoinositol monophosphate; Ins, myo-inositol; PBS, phosphate-buffered saline; cpm, counts per minute; PtdIns(4,5)P₂, phosphatidylinositol (4,5)bisphosphate; BSA, bovine serum albumin; B and B₀, cpm Ins(1,4,5)P₃ in the presence and absence of unlabeled compound, respectively.

of saturable, high affinity binding sites for $\text{Ins}(1,4,5)\text{P}_3$ in microsomal fractions from bovine adrenal cortex (12). A modification of the competition radiobinding assay is commercially available and has recently been applied to quantify the $\text{Ins}(1,4,5)\text{P}_3$ content of slime mold cells (13). Unfortunately, this commercial kit is expensive and requires large numbers of cells. The purpose of the present study was to develop an alternative competition radiobinding assay that would be optimal for the measurement of the $\text{Ins}(1,4,5)\text{P}_3$ content of human leucocytes and changes in it upon stimulation.

MATERIALS AND METHODS

Preparation of cell lysates. Blood collected from healthy donors by venepuncture was subjected to Ficoll-Hypaque centrifugation ($\rho=1.077$; Pharmacia Inc., Uppsala, Sweden) at $440 \times g$ for 20 min at 18°C . After resuspension of the pellet in phosphate-buffered saline (PBS, pH 7.4) the granulocytes were purified by plasmasteril (Fresenius A.G., Bad Homburg, FRG) sedimentation ($1 \times g$) for 10 min at 37°C , washed in PBS and the contaminating erythrocytes were lysed with distilled water. The monocyte- and lymphocyte-enriched preparations were isolated from the Ficoll-Hypaque interphase by elutriation centrifugation with a Beckman J2-21 centrifuge (Beckmann Instruments, Mijdrecht, The Netherlands) as described (14) and resuspended in PBS. In all cell suspensions; viability exceeded 95% as determined by trypan-blue exclusion.

To extract the inositolphosphates from the cells, 100 μl icecold 3.5% vol/vol perchloric acid was added to 100- μl aliquots of 10^8 cells/ml, and after 20 min at 4°C these samples were centrifuged for 1 min at $7,000 \times g$. Next, the supernatants were neutralized with 50 μl saturated sodium bicarbonate for 20 min at 4°C , centrifuged for 1 min at $7,000 \times g$ and the acquired supernatants were stored at -20°C for up to 2 months. Neutralized perchloric acid-treated PBS served as a control.

Stimulation of leucocytes with f-MLP. Leucocytes were exposed to 1 μM formyl-methionyl-leucyl-phenylalanine (f-MLP; Sigma Chemical Co., St. Louis, MO, USA) dissolved in dimethylsulfoxide (DMSO, Serva, Heidelberg, FRG) and diluted in PBS. Stimulation was stopped at selected times (0-300 secs) by the addition of icecold 3.5% perchloric acid. As control, cells were exposed to PBS with an equivalent concentration of DMSO for the same intervals.

Competition radiobinding assay for $\text{Ins}(1,4,5)\text{P}_3$. Microsomal preparations originating from bovine adrenal cortex were obtained as described (12). Briefly, bovine adrenal cortices, dissected free of fat and medullary tissue, were homogenized with a blender (Braun, Frankfurt, FRG) in 20 mM sodium hydrogencarbonate and centrifuged for 10 min at $500 \times g$ to remove large pieces of tissue. The supernatant was centrifuged for 20 min at $25,000 \times g$, and then the pellet was washed and diluted in 20 mM Tris-HCl-buffer (pH 7.5) to a total protein content of about 15 mg/ml. Protein was determined according to Markwell et al. (15), with serial dilutions of bovine serum albumin (BSA) used as standards. $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ (specific activity 20-60 Ci/mmol) and unlabeled $\text{Ins}(1,4,5)\text{P}_3$ were purchased from Amersham International, Amersham, Bucks, UK).

Next, 20 μl cell lysate, 20 μl 0.1 M Tris-HCl-buffer (pH 9) supplemented with 4 mM EDTA and 0.4% BSA, 20 μl of the optimal dilution of $2\text{-}^3\text{H}\text{-Ins}(1,4,5)\text{P}_3$, and 20 μl microsomal preparation were pipetted into an Eppendorf vial. Binding of $\text{Ins}(1,4,5)\text{P}_3$ to components of the microsomal preparation was allowed to continue for 20 min at 0°C . Bound $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ was then separated from free $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ by centrifugation of the mixture for 2 min at $10,000 \times g$, the supernatant was then discarded, and the pellet resuspended in 100 μl PBS and mixed with 1.0 ml insta-gel (Packard Chemical Operations, Groningen, The Netherlands) for liquid scintillation counting for 10 min. The amount of $\text{Ins}(1,4,5)\text{P}_3$ in the cell lysate was quantified by comparison with the

standard curve based on serial dilutions of unlabeled $\text{Ins}(1,4,5)\text{P}_3$ (Amersham) made in saline (range 0 to 2.5 pmol $\text{Ins}(1,4,5)\text{P}_3/20\ \mu\text{l}$). The mean intracellular $\text{Ins}(1,4,5)\text{P}_3$ concentrations were calculated from the amount of $\text{Ins}(1,4,5)\text{P}_3$ and the mean cell volume of the leucocytes (16).

Specific binding values were used to compare the various batches of bovine adrenal cortex microsomal preparation with respect to binding of $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$. To calculate the specific binding, we determined the non-specific binding, i.e., binding of $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ to the microsomal preparation in the excess of unlabeled $\text{Ins}(1,4,5)\text{P}_3$, and total binding, i.e., binding of $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ in the absence of unlabeled $\text{Ins}(1,4,5)\text{P}_3$.

Fractionation of inositolphosphates in granulocyte lysates. Inositolphosphates in lysates of granulocytes were separated on a Dowex AG 1-X8 (formate-form; mesh size: 200-400; BioRad Laboratories, Richmond, CA, USA) as described (Berridge, 1983). The standards were $2\text{-}^3\text{[H]}\text{-Ins}$, $2\text{-}^3\text{[H]}\text{-Ins}(1)\text{P}_1$, $2\text{-}^3\text{[H]}\text{-Ins}(1,4)\text{P}_2$, and $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ (all from Amersham).

RESULTS

Optimalization of the competition binding assay. To determine the optimal conditions for the interaction between $\text{Ins}(1,4,5)\text{P}_3$ and its binding sites in the microsomal preparation, a known amount of $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ was mixed with a fixed amount of microsomal preparation under varying conditions. Binding of $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ to components of the microsomal preparation was maximal after 20 min at 0°C , independent of the pH of the $\text{Ins}(1,4,5)\text{P}_3$ -containing buffer (pH range 7 to 11), but decreased with increasing concentrations of sodium chloride in the buffer (results not shown). In all further experiments $\text{Ins}(1,4,5)\text{P}_3$ was therefore dissolved in saline. Specific binding of $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ was maximal when $20\text{-}\mu\text{l}$ microsomal preparation and about 1200 cpm $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ were used. Under these conditions, median specific and non-specific binding amounted to 30% (range 29%-33%) and 7% (6%-10%), respectively, (four different batches of microsomal preparation; for each batch: $n=12$ experiments).

In connection with the possibility that perchloric acid treatment of cells degraded $\text{PtdIns}(4,5)\text{P}_2$ to form $\text{Ins}(1,4,5)\text{P}_3$, we exposed various amounts of $\text{PtdIns}(4,5)\text{P}_2$, ranging from 10 pmol to 1 nmol dissolved in $100\ \mu\text{l}$ PBS, to $100\ \mu\text{l}$ 3.5% perchloric acid and then neutralized the mixture with $50\ \mu\text{l}$ saturated sodiumbicarbonate. Equal amounts of $\text{PtdIns}(4,5)\text{P}_2$ dissolved in $250\ \mu\text{l}$ PBS served as control. Next, the amount of material competing with $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ for binding to components of the microsomal preparation was determined. No differences were found between the values for perchloric acid-treated and control samples $\text{PtdIns}(4,5)\text{P}_2$ ($n=3$; $p<0.01$). Furthermore, the efficiency of extraction of $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ was higher than 90% (results not shown).

Measurements of the $\text{Ins}(1,4,5)\text{P}_3$ content were highly reproducible, since the intra-assay coefficient of variation ranged from 7 to 18% (four batches of cell lysate; each determined at least in quadruplicate) and the inter-assay coefficient of variation from 12-16% (three batches of cell lysate).

Specificity of the competition binding assay.

To find out whether other compounds besides $\text{Ins}(1,4,5)\text{P}_3$ interact with binding sites in the microsomal preparation, we fractionated a human granulocyte extract on a Dowex AG 1-X8-column and the presence of material competing with $2\text{-}^3[\text{H}]\text{-Ins}(1,4,5)\text{P}_3$ for binding to sites in the microsomal preparation determined. Only the $\text{Ins}(1,4,5)\text{P}_3$ -containing fraction was able to compete with $2\text{-}^3[\text{H}]\text{-Ins}(1,4,5)\text{P}_3$ for binding to such sites (Fig. 1). Further, we investigated the specificity of the competition radiobinding assay by incubating a granulocyte extract together with an optimal dilution of an $\text{Ins}(1,4,5)\text{P}_3$ -5-phosphatase preparation (a generous gift of Dr. C. Erneux, Free University of Brussels, Belgium) for 60 min at room temperature and determined the amount of the cross-reacting activity in the extract with the competition binding assay. This enzyme reduced the cross-reacting activity in the granulocyte extract by 70%, which is comparable to the enzymatical degradation of $^{32}\text{P}\text{-Ins}(1,4,5)\text{P}_3$ (New England Nuclear Research Products, DuPont de Nemours BV, 's-Hertogenbosch, The Netherlands) added to the granulocyte extract, which amounts to 71%.

Addition of high concentrations (up to $1\text{ }\mu\text{M}$) of c-AMP or c-GMP (Amersham), did not affect the binding of $2\text{-}^3[\text{H}]\text{-Ins}(1,4,5)\text{P}_3$ to sites in the microsomal preparation (results not shown). However, 0.5 nmol or more $\text{PtdIns}(4,5)\text{P}_2$ competed with labeled $\text{Ins}(1,4,5)\text{P}_3$ for binding to sites in the microsomal preparation; on a molar basis binding of $\text{PtdIns}(4,5)\text{P}_2$ to such components was about 1000 times lower than that of $\text{Ins}(1,4,5)\text{P}_3$ (Fig. 2). Since the $\text{PtdIns}(4,5)\text{P}_2$ content of human leucocytes lies roughly between 1 and

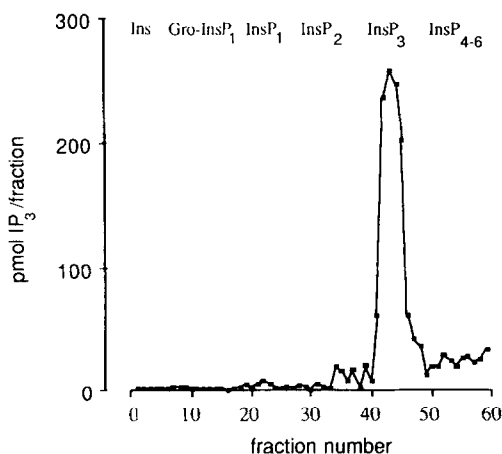


Fig. 1. Competition between the various fractions containing inositolphosphates and $2\text{-}^3[\text{H}]\text{-Ins}(1,4,5)\text{P}_3$ for binding to sites in the adrenal cortex microsomal preparation.

Extracts of human granulocytes were made with icecold 3.5% perchloric acid followed by neutralization with saturated sodium hydrogencarbonate. Inositolphosphates were fractionated on an AG 1-X8 column using Ins , $\text{Ins}(1)\text{P}_1$, $\text{Ins}(1,4)\text{P}_2$, and $\text{Ins}(1,4,5)\text{P}_3$ as standards. All fractions were screened for cross-reactivity in the competition binding assay. Only the fractions containing $\text{Ins}(1,4,5)\text{P}_3$ displayed cross-reactivity.

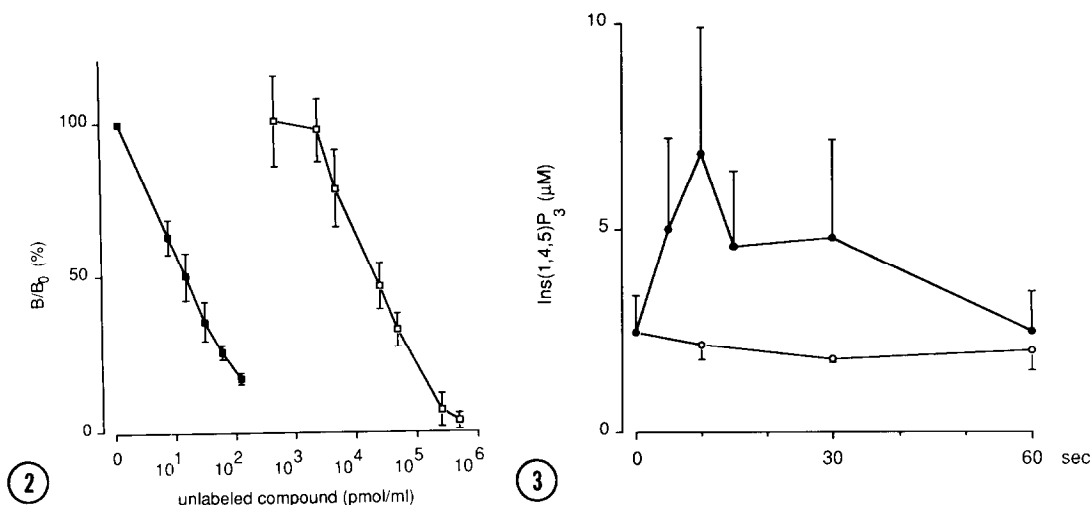


Fig. 2. Binding of $\text{Ins}(1,4,5)\text{P}_3$ or $\text{PtdIns}(4,5)\text{P}_2$ to $\text{Ins}(1,4,5)\text{P}_3$ -binding sites in bovine adrenal cortex microsomal preparation.

Competition between $\text{Ins}(1,4,5)\text{P}_3$ (closed symbols) or $\text{PtdIns}(4,5)\text{P}_2$ (open symbols) and $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ for binding to sites in a bovine adrenal cortex microsomal preparation was determined with the competition binding assay. Cpm bound $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ in the presence of the various amounts of the unlabeled compound (B) and cpm bound $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ in the absence of the unlabeled compound (B_0) were determined. B is expressed as a percentage of B_0 . The results are means and standard deviations of three experiments; each value is based on at least quadruplicate determinations.

Fig. 3. Effect of f-MLP on the $\text{Ins}(1,4,5)\text{P}_3$ content of human granulocytes.

Granulocytes were stimulated with $1\text{ }\mu\text{M}$ f-MLP (closed symbols) or with 10^{-4} M DMSO (control; open symbols) for selected intervals and extracts of these leucocytes were made. The $\text{Ins}(1,4,5)\text{P}_3$ content of the these cell extracts was determined with the competition binding assay. Between 5 and 30 sec after f-MLP stimulation, the values for $\text{Ins}(1,4,5)\text{P}_3$ of granulocytes but not lymphocytes increased ($p < 0.01$) relative to basal values and control values. The values are expressed as the mean and standard deviation for four experiments (measured in quadruplicate).

5 nmol per 1×10^8 cells (based on values for the brains, 17,18), the possibility that the $\text{Ins}(1,4,5)\text{P}_3$ -containing fraction of the cell lysates is contaminated with substantial amounts of $\text{PtdIns}(4,5)\text{P}_2$ was investigated. For this purpose we determined the organic phosphate content of this fraction of a lysate of 2×10^8 granulocytes as described (19). In this concentrated cell lysate the amount of organic phosphate lay below the detection level of 5 pmol (results not shown).

Changes in the intracellular $\text{Ins}(1,4,5)\text{P}_3$ content of blood leucocytes after stimulation with f-MLP. The mean and sd intracellular $\text{Ins}(1,4,5)\text{P}_3$ content of human blood monocytes, granulocytes, and lymphocytes amounted to $3.3 \pm 1.2\text{ }\mu\text{M}$, $3.1 \pm 1.4\text{ }\mu\text{M}$, and $4.6 \pm 1.5\text{ }\mu\text{M}$, respectively. Neutralized perchloric acid-treated PBS did not affect the binding of $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ to the binding sites in the microsomal preparation (results not shown). After stimulation with f-MLP the $\text{Ins}(1,4,5)\text{P}_3$ content of human granulocytes (Fig. 3)

and monocytes (results not shown) increased about 2-3 times within 10 sec and then gradually decreased, returning to basal values at 60 sec. Lymphocytes did not respond to f-MLP with a rise of their $\text{Ins}(1,4,5)\text{P}_3$ content.

DISCUSSION

The present competition binding assay for $\text{Ins}(1,4,5)\text{P}_3$ is rapid, convenient, and does not require loading of the cells with labeled probes or chromatographic separation of inositolphosphates. Since perchloric acid treatment of the cells does not lead to $\text{Ins}(1,4,5)\text{P}_3$ formation from $\text{PtdIns}(4,5)\text{P}_2$ and the recovery of $\text{Ins}(1,4,5)\text{P}_3$ after extraction was excellent, it may be concluded that the $\text{Ins}(1,4,5)\text{P}_3$ content of the cell lysate represented that of intact cells. Measurements of the $\text{Ins}(1,4,5)\text{P}_3$ content of cells performed on the same day as well as on different days were reproducible. The results obtained with different batches of adrenal cortex microsomal preparation can be compared with each other without any correction.

Several lines of evidence point to the specificity of the method. It has been reported that of all inositolphosphates studied only $\text{Ins}(1,4,5)\text{P}_3$ and to a considerably smaller degree some isomers of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ can compete with $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ for binding to its receptor (20). We found that only $\text{Ins}(1,4,5)\text{P}_3$ -containing fractions of a human granulocyte extract, obtained by chromatography on a AG 1-X8-column, were able to compete with $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ for binding to sites in the microsomal preparation. However, other inositoltrisphosphate isomers besides $\text{Ins}(1,4,5)\text{P}_3$ coeluted with this method. The possibility that $\text{Ins}(1,3,4)\text{P}_3$, the major contaminant in the $\text{Ins}(1,4,5)\text{P}_3$ fraction, binds to components of the adrenal cortex microsomal preparation has been excluded, since $\text{Ins}(1,4,5)\text{P}_3$ -5-phosphatase reduced the cross-reactivity of the granulocyte extract to the same extent as it did degrade $^{32}\text{[P]}\text{-Ins}(1,4,5)\text{P}_3$. It cannot be entirely excluded that $\text{Ins}(1,2\text{-cyclic},4,5)\text{P}_3$ is not determined with the assay. However, in the present study this cyclic isomer of $\text{Ins}(1,4,5)\text{P}_3$ probably did not play a role, since resting cells contain only small amounts of this isomer and little is formed after brief stimulation (21). We also found that other second messengers, such as c-AMP and c-GMP, did not inhibit the binding of $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ to the adrenocortical preparation. $\text{PtdIns}(4,5)\text{P}_2$ inhibited the binding of $2\text{-}^3\text{[H]}\text{-Ins}(1,4,5)\text{P}_3$ to the microsomal preparation. This cross-reactivity did not affect the present results, since the neutralized perchloric acid cell extracts do not contain detectable amounts of phospholipid and thus $\text{PtdIns}(4,5)\text{P}_2$.

The values for the $\text{Ins}(1,4,5)\text{P}_3$ content of the various human leucocytes ranged from 3 μM to 5 μM . Values of the same order of magnitude were found for mouse peritoneal and alveolar macrophages as well as for activated peritoneal macrophages (results not shown). The present values are comparable to those obtained for

Dictyostelium discoideum micro-organisms (13) and 3T3 fibroblasts (22) with the competition binding assay, for AR42J pancreatoma cells using the isotopic equilibrium method (3), and for MDCK cells using mass spectrometry of separated, hydrolysed, and derivatised $\text{Ins}(1,4,5)\text{P}_3$ (8). However, the present $\text{Ins}(1,4,5)\text{P}_3$ values are considerably higher than those obtained for human platelets with an enzymatic method (10) and for rabbit peritoneal neutrophils with a different competition binding assay (9). The reasons for divergent $\text{Ins}(1,4,5)\text{P}_3$ values found with the present competition radiobinding assay and that employed by Bradford and Rubin (9) are not clear. One possible explanation for this discrepancy, i.e. the intracellular $\text{Ins}(1,4,5)\text{P}_3$ content of neutrophils from two species differs, can be excluded, since we found that the $\text{Ins}(1,4,5)\text{P}_3$ content of rabbit neutrophils (68 ± 14 pmol $\text{Ins}(1,4,5)\text{P}_3/10^8$ neutrophils) is about the same as the value for human neutrophils.

A great advantage of the present method is the large reduction in costs and number of cells required in comparison to the commercial kit. Since we prepared the $\text{Ins}(1,4,5)\text{P}_3$ -binding material ourselves, which takes about one day to isolate adrenalcortical material sufficient for 8,000 determinations, and diminished the volume of the various components of the binding assay, the price was reduced from about 20 US dollars to 1 US dollar per sample. The number of cells required to prepare a cell lysate was reduced from 1×10^7 to 2×10^6 cells. These advantages led us to quantify the changes in the $\text{Ins}(1,4,5)\text{P}_3$ content of human leucocytes during f-MLP stimulation.

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